Cumulus Cells Apoptosis as an Indicator to Predict the Quality of Oocytes and the Outcome of IVF–ET[1]

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Submitted: February 1, 2001
Accepted: April 18, 2001

Purpose: *Our purpose was to establish an evaluation system for oocyte quality based on the incidence of cumulus cells apoptosis and to examine the effect of coculture, using autologous cumulus cells, on the outcome of IVF–ET according to proliferative activities of helper cells and the incidence of cumulus cells apoptosis.*

Methods: *Cumulus cell masses were collected from 91 mature oocytes among 330 oocytes retrieved from a total of 34 IVF–ET cycles with tubal infertility and unexplained infertility. The incidence of apoptosis in cumulus cells was assessed by apoptosis detection kit fluorescein. On ovum pick up, 2nd day embryos were cocultured with autologous cumulus cells. Prior to coculture, in vitro proliferative activity of cumulus cells was evaluated.*

Results: *Cumulus cells from patient groups over 40 years old had a significantly increased apoptosis incidence, a lower fertilization rate, and the decreased number of oocytes retrieved compared to the other age groups (* $P < .05$*). The incidence of cumulus cells apoptosis was significantly lower when the number of oocytes retrieved was 5 or less (* $P < .05$*). Cumulus cells from fertilized oocytes (0.43 ± 0.07%) and those from patients who became pregnant (0.44 ± 0.11%) following IVF–ET showed a significantly lower incidence of apoptosis compared to those of unfertilized oocytes (1.80 ± 0.35%;* $P < .001$*) and the nonpregnant group (0.81 ± 0.10%;* $P < .05$*). Embryo quality also had a negative correlation with the incidence of cumulus cells apoptosis. Coculture of fertilized oocytes with cumulus cells with high proliferative activity resulted in improved rates of implantation and pregnancy compared to that with poor active cumulus cells. No significant difference was found between the in vitro proliferative activity of cumulus cells and the incidence of cumulus cells apoptosis (* $P < .063$*).*

Conclusions: *The age of women might influence the incidence of apoptosis in cumulus cells, and the increased incidence of apoptosis is associated with the number of oocytes retrieved, the fertilization rate, and the pregnancy outcome following IVF–ET. These results suggest that the incidence of cumulus cells apoptosis can be used in predicting oocyte quality, outcome of IVF–ET, and age-related decline in fertility.*

KEY WORDS: apoptosis; cumulus cells; oocyte quality; outcome of IVF–ET.

INTRODUCTION

Oocytes quality could be the single most important factor in determining successful fertilization and implantation. Oocyte maturity, fertilization rate, and incidence of chromosomal abnormalities in the human oocyte are affected by the ovarian hyperstimulation method used or age of patients undergoing IVF–ET (1–3). The decreased rate of pregnancy in IVF–ET is associated with the increase in percentage of meiotic competence failure oocytes (4). These reports indicate that hyperstimulated oocytes are of various qualities and that obtaining good quality oocytes is very important for successful IVF–ET.

Unfortunately, an accurate system to evaluate oocyte quality is lacking (5,6). The most convenient evaluation system for oocyte quality is based on oocyte morphology and status of oocytes–cumulus complexes. This evaluation method could be imprecise

[1] This paper was presented in part as a poster at the Conjoint Annual Meeting of American Society for Reproductive Medicine and Canadian Fertility and Andrology Society, Toronto, Ontario, Sept. 25–30, 1999.

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1058-0468/01/0900-0490$19.50/0

and subjective since there is no clear correlation between oocyte quality and outcome of IVF–ET like fertilization rate and pregnancy rate (7,8).

Apoptosis is defined as programmed cell death for homeostasis and is closely involved with most of the reproductive processes, including follicular atresia (9). Seifer *et al.* (10) reported that apoptosis can be used to estimate a function of ovarian reserve in women undergoing in vitro fertilization (IVF). Cumulus cells surround and intercommunicate with oocytes during follicular development and after ovulation, suggesting that the incidence of apoptosis in cumulus cells could influence oocyte quality.

Cumulus cells have recently been used as helper cells for coculture of human embryos (11,12). However, information concerning the effect of coculture based on status of cumulus cells, particularly those relating to the incidence of apoptosis or their proliferative activity in vitro, is limited.

This study was performed to establish an evaluation system for oocyte quality based on the incidence of cumulus cells apoptosis and to investigate the effect of coculture, using autologous cumulus cells, on the outcome of IVF–ET according to proliferative activities of helper cells and the incidence of cumulus cells apoptosis.

MATERIALS AND METHODS

The study was approved by the Institutional Review Board of Pusan National University Hospital.

Subjects

This study was performed in a total of 34 consecutive cycles from 33 women who underwent IVF–ET with tubal obstruction (23 cycles) and unexplained infertility (11 cycles) at the Pusan National University Hospital (PNUH) from August 1997 to July 1998. All women who participated in the study gave written informed consent. The consent form and protocol were approved by the Human Investigation Committee of PNUH. The mean age of the patients was 33.2 $\pm$ 5.0 years (mean $\pm$ SD, ranging from 26 to 44 years).

Ovarian Hyperstimulation and IVF Procedures

Controlled ovarian hyperstimulation was performed by a long standard protocol with gonadotropin releasing hormone agonist (buserelin acetate; Frankfrut, Germany)/human menopausal gonadotropin (hMG; Organon, Netherlands)/highly purified follicle stimulating hormone (HP-FSH; Serono; Norwell). Briefly, buserelin acetate was given daily at a dose ranging from 0.1 to 0.05 mL for a minimum of 2 weeks, after which serum E_2 was obtained to assess whether adequate suppression had been achieved. When the serum E_2 level was 50 pg/mL (conversion factor to SI unit, 3.671), one or two ampules of hMG and one or two ampules of 75 IU of HP–FSH were given IM in the morning and evening in addition to the daily dose of buserelin acetate.

Follicular development was assessed in all patients by monitoring serum E_2 level and by ovarian ultrasonography. Human chorionic gonadotropin, hCG (pregnyl; Organon, Netherlands), was administered when the estradiol level reached the maximal peak and at the same time, at least two dominant follicles were 17 mm or larger. Regarding the ultrasonographic features of oocyte before retrieval, most of follicles were round and compact, and looked healthy.

Oocyte retrieval by transvaginal ultrasonographic guidance was performed approximately 34–35 h after the hCG administration. Follicular aspirates were transferred into 60 mm tissue culture dishes (Falcon 3002; Becton Dickinson and Company, Lincoln Park, NJ). Oocyte–cumulus cells (OCC) complexes were isolated under a dissecting microscope (SZH, Olympus, Tokyo, Japan). Maturity and quality of each OCC-complex were graded under inverted phase contrast microscope (Olympus IX 70, Tokyo, Japan) by spreading each OCC-complex to one line on the surface of a 60 mm culture dish. Ooyctes with polar bodies and extensive cumulus cells were regarded as mature. Cumulus cells were removed from 3 or 5 OCC-complexes when the number of OCC complexes were $\geq$10, or from 2 or 3 oocytes when the number were $<$10, and subsequently, used in the experiment.

Four or five oocytes were placed into each organ culture dish (Falcon 3037; Becton Dickinson and Company) containing 0.9 mL of IVF-M (Medicalt, Cophenhagen, Denmark) medium. Highly motile spermatozoa were collected by a discontinuous percoll gradient (100–90–50%) method and were used for insemination 4–12 h after oocyte retrieval, depending on the oocyte maturity. Fertilization was confirmed 16–20 h after insemination by visualization of two pronuclei. Fertilized oocytes were transferred to fresh IVF-M medium (1 mL), and then cultured for 24 h or more, followed by coculture with autologous cumulus cells. After coculture, embryos were graded

based on the size of blastomere and the presence of fragmentation, using Bolton's definition (13), just before embryo transfer (ET). Grade A was the best quality embryo and Grade D was the worst with Grade B to Grade C in between. The embryo transfer was performed with a Wallace Catheter (Simcare, Peter Road lancing, WS, UK) 3 days after oocyte recovery.

Coculture of Embryos Using Autologous Cumulus Cells and Evaluation of the In Vitro Proliferative Activity of Cumulus Cells

When oocytes were checked for fertilization on the first day after oocyte retrieval, cumulus cells were attached and dispersed to the bottom of the insemination dish in a monolayer. This study used cumulus cells as the helper cells for coculture using the following procedure. In brief, after transferring fertilized oocytes into fresh culture medium, the remaining cells attached at the bottom of the dish were washed two to three times to remove any cell debris including spermatozoa and then were cultured in fresh Ham's F-10 medium supplemented with 10% human fetal cord serum (hFCS) for 20 h. On the next day, cumulus cell monolayers were washed again one to two times in fresh Ham's F-10 + 10% hFCS. Subsequently, the in vitro proliferative activity of cumulus cells was divided into two groups based on the degree of morphological change and proliferation. As shown in Fig. 1, Group I showed the extensive morphological change and proliferation in vitro. Group II had less morphological change, and minimal proliferation, or appeared as clumps.

Assessment of Apoptosis in Cumulus Cells

After follicle aspiration and evaluation of oocyte maturity, a small portion of cumulus cell masses was removed from 2–5 mature oocyte–cumulus complexes per cycle, using a pasteur pipette and placed into a new culture dish (Falcon 3037; Becton Dickinson and Company), containing 0.9 mL of Ham's F-10 medium. Hyaluronidase (Sigma, St. Louis, MO) was added to the dishes containing cumulus cell masses at a final concentration of 0.01% (wt/vol). The cell masses were separated into single cells by vigorous pipetting for 5–10 min with a micropipette of less than 100 μm in pore size diameter. After centrifugation (Kubota 2100, Tokyo, Japan) at 300 $\times$ g for 5 min, the supernatant was removed, and the cell pellet was resuspended in remaining medium (about 20 μL) by mouth pipetting for about 1 min or more for complete separation into single cells. The cell suspension was spread onto two glass slides and then air-dried. After fixing in 4% paraformaldehyde in PBS for 15 min, the cumulus cells were stained with the Apotag In Situ Apoptosis Detection Kit Fluorescein (Oncor, Gaithersburg, MD) following the manufacture's procedure. Apoptotic cells show an intense yellow fluorescence, whereas normal cells appear red when stained with propidium iodine (Fig. 2). The apoptotic cells were identified and counted randomly among 200 cumulus cells at 1,000 $\times$ Magnification.

Statistical Analysis

All data for incidence of cumulus cells apoptosis were presented as a mean $\pm$ SEM. Statistical analysis was carried out using one-way ANOVA analysis, the unpaired student t test, and chi-square test. $P < .05$ was considered statistically significant.

RESULTS

Three hundred-thirty-three oocytes were retrieved from 34 cycles controlled ovarian hyperstimulated for IVF–ET. Among them 2–4 mature oocytes with morphologically good quality per cycle were selected for the assay of apoptosis of cumulus cells to exclude the variation of apoptosis incidence with the maturity of oocyte. As a result, a total of 91 oocytes were provided in the assessment of cumulus cell apoptosis.

As patient age is a crucial factor that may influence oocyte quality and outcome of IVF–ET, the incidence of apoptosis in cumulus cells was studied at various ages. As shown in Table I, the incidence of apoptosis was significantly higher in patients over 40 years old (1.59 $\pm$ 0.23%) compared to 0.61 $\pm$ 0.10% in patients aged $\leq$30 years old, 0.75 $\pm$ 0.13% in patients 31–35 years old, and 0.75 $\pm$ 0.20% in patients 35–40 years old ($P < .005$). Fertilization rate was significantly decreased in patients over 40 years old (40.0 $\pm$ 24.5%) compared to other age groups ($P < .05$). These results suggest that the incidence of apoptosis in cumulus cells and oocyte fertilization rates were influenced by the IVF patient's age, and that both of these factors may be closely related.

Age of patients undergoing IVF–ET was also associated with the number of oocytes retrieved. The age was significantly older when the number of oocytes retrieved was 5 or less as compared to other group with more than 5 oocytes retrieved ($P < .05$). The incidence of apoptosis in cumulus cells was 1.13 $\pm$ 0.19%,

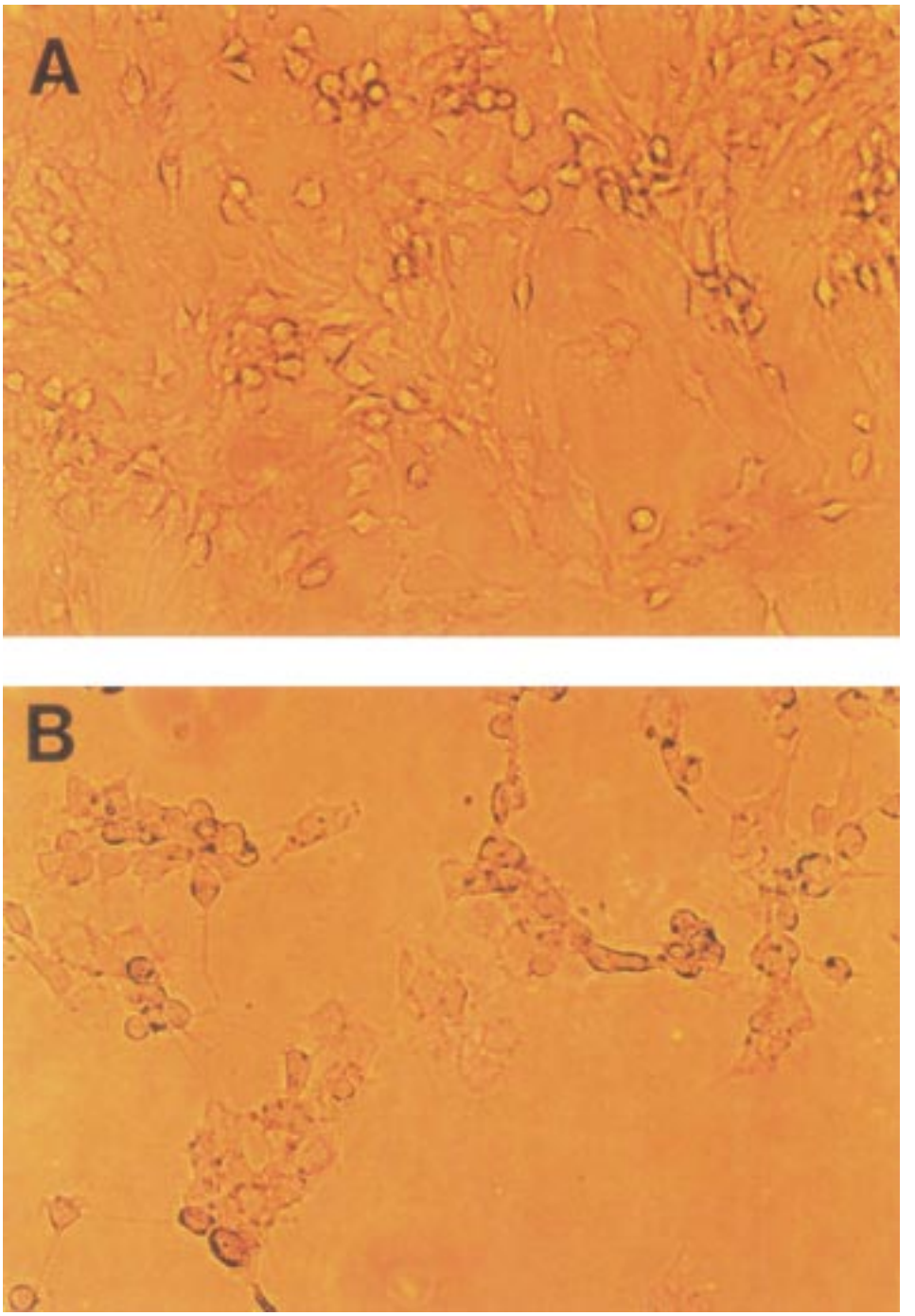

Fig. 1. Phase contrast microscopic morphological changes and proliferation in cumulus cells that will be used in coculture, 2 days after oocyte retrieval. (A) Extensive morphological changes and proliferation (Group I), (B) No or minimal morphological change (Group II). Magnification × 200.

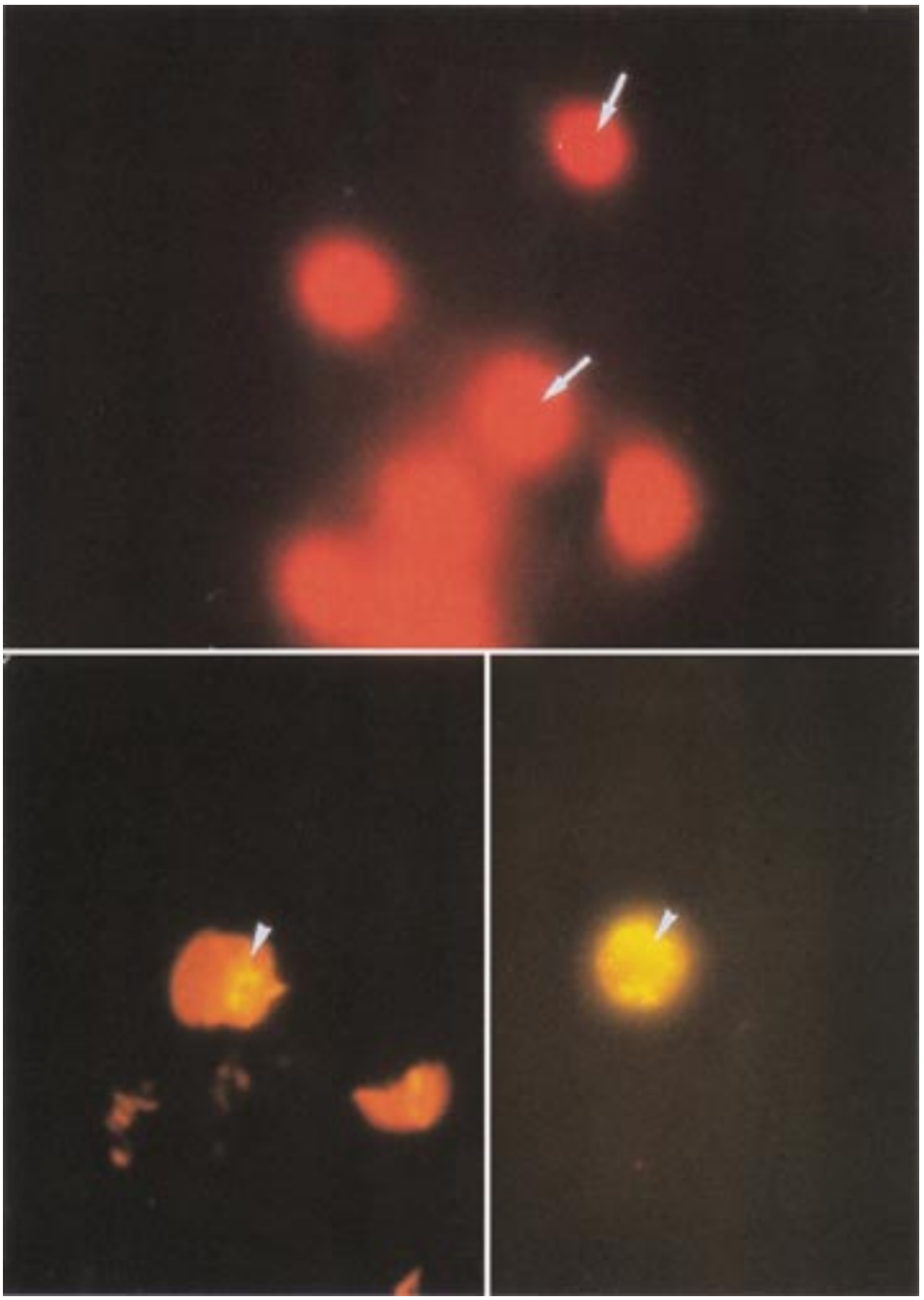

Fig. 2. Immunofluorescent microscopic detection of apoptosis in cumulus cells stained with apoptosis detection kit (Oncor). Magnification × 1,000. Apoptotic cumulus cells show intense yellow fluorescence (arrowhead), whereas normal cells appear redish color stained with propidium iodide (arrow).

0.63 ± 0.12%, and 0.77 ± 0.11% when the number of oocytes retrieved was 5 or less, 6–10, and >10, respectively. There was a significant difference between the number of oocytes retrieved and the incidence of cumulus cells apoptosis ($P < .05$). However, fertilization rate was not significantly different when compared to the number of oocytes retrieved (Table II).

Of 91 oocytes provided in the assay for cumulus cells apoptosis, 28 oocytes were unfertilized. The

Table I. Comparison of the Incidence of Cumulus Cells Apoptosis with the Age of Patients

Age (years)	No. of cycles	No. of oocytes		Fertilization rate (%)	Incidence of apoptosis (%)
		Retrieved	Used for assay		
≤30	13	151	36	88.4 ± 4.4	0.61 ± 0.10
31–35	13	105	32	63.4 ± 11.4	0.75 ± 0.13
36–40	4	43	12	72.8 ± 14.1	0.75 ± 0.20
≥41	4	31	11	40.0 ± 24.5[a]	1.59 ± 0.23[b]

Note. All data are represented as mean ± SEM.
[a] $P < .05$ (vs. other groups).
[b] $P < .005$ (vs. other groups).

incidence of apoptosis in cumulus cells from fertilized oocytes was 0.43 ± 0.07%, which was significantly lower compared to that of unfertilized oocytes (1.80 ± 0.35%; $P < .001$).

To investigate whether apoptosis in cumulus cells affects embryo quality, incidences of apoptosis for 63 fertilized embryos were compared to embryo quality. The results showed that there was significant difference between these two factors. The incidence was 0.24 ± 0.06% for Grade A embryos, 0.42 ± 0.12% for Grade B embryos, and 1.18 ± 0.23% for Grade C embryos. There were no Grade D embryos after coculture (Table III).

Of 34 IVF–ET cycles, 10 patients were pregnant, and the pregnancy rate per cycle was 29.4%. The fertilization rates were significantly higher in the pregnant group (93.3 ± 2.9%) than in the nonpregnant group (62.2 ± 0.12%; $P < .05$). The incidence of apoptosis in cumulus cells also decreased by twofold in the pregnant group (0.44 ± 0.11%) compared to the nonpregnant group (0.81 ± 0.10%; $P < .05$). However, the patient's age, the number of oocytes retrieved, and the number of embryos transferred per cycle showed no significant difference between the pregnant and the nonpregnant groups (Table IV).

In coculture, using autologous cumulus cells, the implantation and pregnancy rates were compared to in vitro proliferative activity and morphological changes. In highly active cumulus cells (Group I), the implantation and pregnancy rates were 12.1 and 40.0%, which were significantly higher than in poorly active cumulus cells (Group II) (6.8 and 21.1%, respectively; $P < .05$). However, other IVF–ET parameters were not related to the in vitro activity of cumulus cells (Table V). The incidences of apoptosis in cumulus cells derived from Group I and Group II were 0.56 ± 0.13% and 0.86 ± 0.10%, respectively ($P = .063$).

DISCUSSION

It is important to accurately assess oocyte quality as well as to obtain good quality oocytes for successful pregnancy following IVF–ET. Apoptosis has been closely associated with follicular atresia (9). Apoptosis in granulosa cells was suggested as a function of ovarian reserve in women undergoing IVF (10). Thereafter, Nakahara *et al.* (14) used the incidence of apoptosis in granulosa cells for the evaluation of oocyte quality. However, several points were raised in their interpretation of the data (15). First of all, it is unreasonable to directly conclude that the relationship between the incidence of granulosa cell apoptosis and oocyte competency exists because they analyzed pooled follicular aspirates on per patient basis and multiple follicles are aspirated per patient per cycle. Secondly, they did not seem to consider the fact that the incidence of apoptosis and oocyte quality can be altered by the age of patient (10,16). In contrast, this study evaluates oocyte quality based on the

Table II. Incidence of Apoptosis According to the Number of Oocytes Retrieved

No. of oocytes retrieved	No. of cycles	Age (mean)	No. of oocytes used for assay	Fertilization rate (%)	Incidence of apoptosis (%)
≤5	10	35.3[a]	23	64.3 ± 13.2[a]	1.13 ± 0.19[a]
6–10	9	30.2	23	70.4 ± 11.5	0.63 ± 0.12
>10	15	31.8	45	72.9 ± 8.5	0.77 ± 0.11
P-value		<.05		.84	.07

Note. All data are represented as mean ± SEM.
[a] $P < .05$ (vs. other groups).

Table III. Incidence of Apoptosis According to the Embryo Grade

Grade of embryo[a]	No. of oocytes used for assay	Incidence of apoptosis (%)
A	40	0.24 ± 0.06
B	15	0.42 ± 0.12
C	8	1.18 ± 0.23[b]
D	0	0

[a] A: equal blastomere and no fragmentation, B: unequal blastomere and no fragmentation, C: partial equal blastomere and small fragmentation (less than 30%), D: unequal blastomere, large fragmentation (more than 30%).
[b] $P < .001$ (vs. other groups).

incidence of apoptosis in cumulus cells derived from each oocyte. In this respect, our evaluation system might be more effective in estimating the quality of each oocyte.

It is well known that the pregnancy rate following IVF–ET decreases as women's age increases (17). These results support that the age of patients undergoing ART is a very important factor for pregnancy (18). The possible reasons for this age-related decline in fertility are oocyte degeneration (19,20) and the reduction of endometrial receptivity (21). The presence of nuclear maturation arrest and DNA fragmentation in ovulated oocytes from aged mice (16,22) strongly supports that female fertility decreases with age due to poor oocyte quality.

This study also show that the incidence of cumulus cell apoptosis significantly increased in patients over 40 years old, whereas the fertilization rate was greatly decreased in that age group. Furthermore, the incidence of apoptosis in cumulus cells influences significantly the embryo quality, the fertilization rate, and the pregnancy outcome following IVF–ET. These results suggest that the incidence of cumulus cells apoptosis could predict the age-related decline in fertility as well as oocyte quality. The greater incidence of cumulus cells apoptosis with reproductive aging may induce unfavorable environments for follicular oocyte development, which results in deterioration of oocyte quality, thus reducing fertilization rates and embryo development.

Table IV. Comparison of Parameters Between Pregnant and Nonpregnant Group Following IVF–ET

	Pregnant	Nonpregnant	*P*-value
No. of cycles	10	24	.115
Age (mean)	30.5	33.2	.114
No. of oocytes retrieved/ cycle	10.2	9.5	
No. of oocytes used for assay	27	64	
Fertilization rate (%)[a]	93.3	62.2	<.05
Incidence of apoptosis (%)[a]	0.44 ± 0.11	0.81 ± 0.10	<.05
No. of embryos transferred/ cycle[a]	4.7 ± 0.6	5.2 ± 0.5	.561

[a] Data are mean ± SEM.

The incidence of cumulus cell apoptosis was significantly higher in unfertilized oocytes than in fertilized oocytes. Most (87%) of the fertilized oocytes were developed to upper grade quality embryos. However, no difference was found in the incidence of cumulus cells apoptosis according to embryo quality. These results imply that most of the oocytes with high incidence of cumulus cell apoptosis might fail to fertilize, and that quality of the developed embryos might not be related to the incidence of cumulus cell apoptosis.

Nakahara *et al*. (14,23) reported that the incidence of apoptosis in cumulus cells rather than granulosa cells is not an appropriate indicator of oocyte quality. The discrepancy of that observation with our result may be due to the different detection methods used to determine apoptosis. They observed fragmented, condensed nuclei of granulosa cells and cumulus cells by H33258 staining method. We also had tested the apoptotic bodies of cumulus cells using H33258. Unfortunately, we could not clearly distinguish the apoptotic bodies. So we used an apoptosis detection kit that recognizes the 3′-OH ends of DNA fragments induced by apoptosis.

Another difference found between the two studies is the relationship between the number of oocytes retrieved and the incidence of ovarian cells apoptosis. Nakahara *et al.* (14) claimed that the incidence of granulosa cell apoptosis increases as the number of oocytes retrieved decreases, and this diminished oocyte number is due to the increased number of empty follicles resulting from the apoptosis in granulosa cells during follicular development.

However, in this study, no correlation was observed between the number of oocytes retrieved and the incidence of cumulus cell apoptosis. Cumulus cells enclose oocytes during all of the follicular maturation and ovulation, unlike granuolsa cells. If an oocyte falls to atresia, it might disappear during follicular development. As a result, cumulus cells are not collected from OCC complexes of atretic follicles at oocyte retrieval. Thus, the incidence of apoptosis of cumulus cells is not associated with the reduction of oocyte number resulting from follicular atresia.

Coculture has been widely used as an attempt to improve in vitro culture conditions over the last 10 years (24–26). Palchot *et al*. (27) suggested that healthy

Table V. Relationships Between the Status of Cumulus Cells and the Outcome of IVF–ET

Status of cumulus cells	Group I	Group II	*P*-value
Age (mean)	31.3	32.3	.502
No. of cycles	15	19	
No. of oocyte retrieved/cycle	9.0	10.3	.426
Fertilization rate (%)	74.2	72.8	.905
Embryo grade			
A	48/470 (68.6%)	63/97 (64.9%)	
B	12/70 (17.1%)	16/97 (16.5%)	
C	8/70 (11.4%)	14/97 (14.4%)	
D	2/70 (0.03%)	4/97 (0.04%)	
No. of embryos transferred/cycle[a]	4.6 ± 0.7	4.9 ± 0.3	.487
Implantation rate (%)[b]	12.1	6.8	<.05
Pregnancy rate/cycle (%)[c]	40.0	21.1	<.05

[a]Data are mean ± SEM.
[b]Defined as the total number of visualized gestation sacs with fetal cardiac activity divided by the total number of embryos transferred in each treatment group.
[c]Clinical pregnancy defined as visualization of an intrauterine sac with ultrasonography.

helper cells are necessary for the beneficial effects of coculture. Watson *et al*. (28) reported that a definite characteristic of somatic helper cells could influence the effect of coculture. Furthermore, the lack of proliferative activity of cumulus-corona cell complex in IVF–ET was suggested as a prognostic indicator for failure of implantation (29). These results support our finding that IVF–ET outcome is different with in vitro proliferative activity of cumulus cells used in coculture. However, there is no clear relationship between the incidence of cumulus cell apoptosis and the in vitro proliferative activity of cocultured cumulus cells. This could be due to the fact that the incidence of apoptosis is mostly less than 1%, and the incidence seems not to influence the effect of coculture directly.

As another reason for the different result of coculture with the proliferative activity of cumulus cells, we can consider the differences in the production of embryotrophic factors by helper cells. Because the status of helper cells is very important for the beneficial effect of coculture as the type and amount of embryotrophic factors critically depend on the types of helper cells (30).

In conclusion, the age of the patient might influence the incidence of apoptosis in cumulus cells, and that the increased incidence of apoptosis is associated with the response to ovarian hyperstimulation, the outcome of fertilization and pregnancy following IVF–ET. These results suggest that the incidence of apoptosis in cumulus cells can be used to predict oocyte quality, outcomes of IVF–ET, and age-related decline in fertility. In addition, the effect of coculture depends on the in vitro proliferative activity of cumulus cells.

ACKNOWLEDGMENTS

This work was supported in part by the 1998 Pusan National University Hospital research grant.

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